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Intranasal Immunization of Pigs with Porcine Reproductive and Respiratory Syndrome Virus like Particles Plus 2', 3' –cGamp Vaccigrade™ Adjuvant Exacerbates Viremia After Virus Challenge

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INTRANSAL IMMUNIZATION OF PIGS WITH PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS LIKE PARTICLES PLUS 2', 3'-cGAMP VacciGrade™ ADJUVANT EXACERBATES VIREMIA AFTER VIRUS CHALLENGE

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This thesis is approved as a creditable and independent investigation by a candidate for the Master of Science degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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ABBREVIATIONS

2’3’-cGAMP = cyclic [G(2’,5’)pA(3’,5’)p]

BTV = Blue Tongue Virus

cDNA = Complementary DNA

CD = Cluster of Differentiation

DIVA = Differentiating Infected from Vaccinated Animals

DNA = Deoxyribonucleic Acid

DPC = Days Post Challenge

DPV = Days Post Vaccination

ER = Endoplasmic Reticulum

GP = Glycoprotein

HPV = Human Papillomavirus

IFN = Interferon

IL = Interleukin

LDV = Lactate Dehydrogenase Elevating Virus

mRNA = Messenger ribonucleic acid

N = Nucleocapsid

nsp = Non-structural Protein

ORF = Open Reading Frame

PAMS = Porcine Alveolar Macrophages

PRRS = Porcine Respiratory and Reproductive Syndrome

PRRSV= Porcine Respiratory and Reproductive Syndrome Virus

PRRSV-MLV = Porcine Respiratory and Reproductive Syndrome Virus – Modified Live Vaccine

RNA = Ribonucleic Acid

SAVE = Synthetic Attenuated Virus Engineering

SDSU = South Dakota State University

sgmRNA = Subgenomic Messenger Ribonucleic Acids

SIRS = Swine Infertility and Respiratory Syndrome

SS =Single Stranded

TGEV = Transmissible Gastroenteritis Coronavirus

USD = United States Dollars

VLPs = Virus Like Particles

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ABSTRACT

INTRANSAL IMMUNIZATION OF PIGS WITH PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS LIKE PARTICLES PLUS 2', 3'-cGAMP VacciGrade™ ADJUVANT EXACERBATES VIREMIA AFTER VIRUS CHALLENGE

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Porcine reproductive and respiratory syndrome virus (PRRSV) is an economically devastating virus that causes a persistent infection and spreads quickly. The virus causes abortions in sows and respiratory diseases in young piglets. Current PRRSV vaccines appear to provide limited cross-protection. A virus-like particles (VLPs) vaccine is made up of key PRRSV structural proteins, which could be cross protective among the different strains. We tested PRRSV VLPs as a vaccine candidate with a 2', 3'-cGAMP VacciGrade™ adjuvant against pigs infected with PRRSV after intranasal immunization to evaluate the immune response and protective efficacy by the VLPs and adjuvant. The VLPs contain PRRSV nucleocapsid (N), glycoprotein 5 (GP5), membrane (M), and the envelope (E) proteins. There were 3 groups of pigs vaccinated with PBS for a control group, a VLPs group, and VLPs group with the 2'3'-cGAMP adjuvant. Vaccination occurred once initially and again as a boost after 2 weeks. Challenge with PRRSV occurred 2 weeks post boost. At 0 days post challenge (DPC), no GP5 or N PRRSV antibodies were found in all animal groups, although N PRRSV antibodies were detected in all groups at 10 DPC. The adjuvant group demonstrated a significantly increased

viremia at 7 and 10 DPC likely due to its ability to enhance the innate immune response and bring more immune cells for the virus to target. Interferon-alpha concentrations were also higher in the VLPs with adjuvant group, but there was only a slight increase in IL-10 and interferon-gamma. The VLPs with adjuvant group showed a significant increase in VLP specific IgG and IgA at 7 DPC when compared to 3 DPC, but they did not reduce viremia. To further test the VLPs as a vaccine candidate, different PRRSV proteins should be incorporated into the VLPs assembly and other routes of vaccination could be considered such as intramuscular injection. A higher dosage of the VLPs could also be tested.

Chapter 1: Introduction and Background

Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) is the infectious agent of PRRS. PRRSV is a host specific virus and infects neonates, piglets, sows, and boars with varying health impacts [44]. Reproductive failure in sows and respiratory distress in young piglets are the major clinical symptoms of PRRS. Sows are known to have abortions, produce stillborn piglets, or farrow pre-mature piglets upon PRRSV infection. Pigs infected with PRRSV become more susceptible to secondary bacteria and/or viral infections and develop high fevers [33, 121]. Although both modified live and inactivated vaccines are commercially available, effective control and elimination of the disease is proving to be a challenge primarily due to the high heterogeneity of the virus. PRRS causes more economic loss in the swine industry than most other viral pig diseases [73]. Researchers strive to find more effective vaccines for the prevention and control of the devastating disease [41].

1. Porcine Reproductive and Respiratory Syndrome Virus

PRRSV is a positive-sense, non-segmented, and single-stranded RNA virus [27, 119, 120]. Its viral genome is about 15 kb in length [27, 61, 94]. PRRSV, a member of the *Arteriviridae* family and *Nidovirales* order, is recognized as two genotypes [94]. The first genotype discovered was known as the Lelystad virus, in representative of the European PRRSV or Type 1 [98]. The second genotype, Type 2, was identified as the North American PRRSV, although it was first referred to as SIRS [6, 17]. Both genotypes are assumed to originate from one source, but the source remains unknown at this time. Other RNA viruses in the *Arterviridae* family include Equine Arteritis Virus (EAV),

Simian Hemorrhagic Fever Virus (SHFV), and Lactate Dehydrogenase-Elevating Virus (LDV) [18, 37, 60].

PRRSV encodes RNA dependent RNA polymerase (RdRP) for its genome replication. Since RdRP lacks the proof-reading mechanism, PRRSV has a high mutation rate during virus replication [28, 39]. Additionally, recombination within the genotypes upon co-infection can occur, which further increases the genetic diversity of PRRSV. The vast genetic diversity of PRRSV presents challenges for disease elimination and prevention through the use of traditional vaccines [48, 91, 108].

PRRSV encodes seven structure proteins and 14 non-structural proteins (nsps) [27]. ORF1a and 1b are translated into two polyproteins (1a and 1ab) that are subsequently cleaved into 14 nsps. The nsps are early proteins responsible for virus replication and gene transcription [27, 85, 94]. Glycoproteins (GP) 2, 3, 4, and 5 are encoded by ORFs 2, 3, 4, and 5 respectively. ORF6 and 7 encode for the membrane protein (M) and nucleocapsid protein (N). ORF2b encodes the envelope protein [18, 37]. The envelope protein is involved in viral attachment, entry and uncoating of the viral genome in the host cells [90]. The nucleocapsid proteins are associated with the RNA genome and form the core of virion. GP2, 3, and 4 form heterotrimers outside the envelope that bind to the extracellular glycoprotein CD163 on porcine alveolar macrophages (PAMS). M and GP5 form heterodimers known as the M/GP5 complex [23]. Each viral protein is important to the viral pathogenesis and spread of more virion particles.

PRRSV Transmission and Pathogenesis

To infect a host cell, PRRSV first must attach to a susceptible host cell [25]. Sialoadhesion and heparin sulfate are two known receptors responsible for the attachment of PRRSV virus to host cells [29, 103]. Binding of GP5 to sialoadhesion is an important step responsible for initiating clathrin-mediated endocytosis or entry into the host [46, 71, 103, 104]. Heparin sulfate serves as a receptor for the M/GP5 complex that permits attachment of the virus to the host cell [25]. Upon entry of the host cell, the binding of GP2 and GP4 to the CD163 receptor allows the virus to release its viral genome into the cytoplasm of the host [22, 106]. This binding is responsible for attachment and entry for both genotypes of PRRSV [22, 106, 110]. Using the host cell's machinery, the open reading frames are translated to express both structural and non-structural proteins [18, 27, 37]. The N protein is responsible for forming the nucleocapsid by binding to the replicated genomic RNA [99, 117, 118]. The newly formed viruses exit the cell through exocytosis [23].

PRRSV can spread through various modes of transmission, which increases the risk of introduction to surrounding herds and presents a challenge to the control and elimination of the virus [81]. Pileri and Mateu recently reviewed the common methods of PRRSV transmission and listed the factors that influence PRRSV transmission and susceptibility [83]. PRRSV can be transmitted vertically from parents to offspring or transmitted by direct contact such as oral or nasal ingestion, sexual transmission, or percutaneous exposure to an infectious object or fluid [14, 24, 43, 79, 80, 97, 109]. PRRSV infections can last many years within a herd because of the virus persistence in some animals [32, 77].

Pigs that are persistently infected with PRRSV can shed virus for longer periods of time, which increases the exposure time of susceptible pigs to the virus [45, 105]. According to Pileri and Mateu's review, susceptibility from shedding virus varies among virus genotypes, ages of the pigs, infectious dose, and the method of transmission [83]. Younger piglets experience longer infectious periods of viremia than older pigs and have higher viral loads. Genotype 2 has also been correlated with higher viral loads [14, 45]. The epidemiological studies of PRRSV help determine necessary biosecurity measures for the control and prevention of PRRSV.

Host Immune Response to PRRSV

The innate immune system is activated shortly after infection, because PRRSV infects macrophages and dendritic cells which are both capable of phagocytosis during an innate response. Upon infection, both of these cells will induce a cytokine response, specifically type 1 interferon, α and β [54]. Interferon (IFN)-α is usually detected at low concentrations both in vitro and in vivo [11, 107]. Further evidence has proved that the cytokine production is higher in transcription of the genes than the ability to translate and produce the interferon proteins. This indicates that PRRSV is actively downregulating the translation of the innate cytokine response and preventing a stronger immune response [62, 124]. This down regulation is linked to the inability of the pig to activate a strong T cell response and the ability to induce an IFN-gamma response [64, 100]. The innate immune response suggests an adjuvant coupled with a vaccine may be necessary to enhance the molecules responsible for activating the acquired immune response [54].

Development of a successful vaccine requires knowledge about how the host's immune system responds to the virus infection. C. L. Loving suggests that pigs are

capable of recognizing PRRSV and developing an immune response that can eliminate the virus. However, the time it takes for the immune response to be initiated is too long, which allows a significant amount of time for transmission of the virus to other susceptible pigs in the herd [54]. Therefore, a vaccine needs to enhance the immune response to provide timely feedback and eliminate the virus before it can spread. A successful vaccine should elicit both humoral and cell mediated immune responses. The humoral response will help to clear the free virus in the blood circulation and fluids; while the cell mediated response will kill PRSSV infected cells.

The role of the cell-mediated response in PRRSV infection is not well known, but T-cells are considered to play an important role in fighting the infection. CD8+ cytotoxic T cells increase around 28 days post PRRSV infection [1, 31]. Although some studies show support of CD8 CTL in virus clearance, others found no relation of CD8 cytotoxic T cells and their ability to clear the infection [34, 52]. Meanwhile, CD4+ cells, responsible for cytokine production and activation of B cells, fluctuates throughout infection based on in vivo and in vitro studies [75, 92]. CD4 and T helper cells provide an interesting view on the ability to activate other immune cells and further research should be encouraged.

The antibody-mediated response against PRRSV is well characterized. [54]. Serum antibody production starts as early as 7 days post infection; however, neutralizing antibodies appear around 28 days post infection [47]. IgM antibodies are the first to appear followed by IgG which is consistently found throughout the persistent infection [51]. Both IgM and IgG are detected prior to the appearance of the virus neutralizing antibody [53]. The anti-N antibody response is the first to be detected and is used in

diagnosis of PRRSV [10]. M protein specific antibody is detected next and followed by GP5 specific antibody [51]. The N protein does not induce neutralizing antibody production, but neutralizing epitopes have been found on the GP4 and GP5 proteins [122].. Osorio, et al, demonstrated the success of using neutralizing antibodies to eliminate or clear PRRSV infection [78]. Other researchers have suggested additional boosts could induce neutralizing antibodies sooner to increase the vaccine's effectiveness [101, 126]. Loving reviewed the available data and concluded that if neutralizing antibodies are produced and available prior to PRRSV exposure, regardless of how the neutralizing antibodies were achieved, then the pig can clear the infection [54]. More research is needed to understand the complete role of neutralizing antibodies and how neutralizing antibodies are induced.

2. PRRSV Vaccine Research Status

Because of the continuing economic burden caused by PRRSV infection [41], researchers are searching for the most efficient and safe vaccine approach that can be used worldwide. Porcine Respiratory and Reproductive Syndrome Virus – Modified Live Virus (PRRSV-MLV) is the most widely used commercially available vaccines. Modified live attenuated viruses are used for vaccination, because the virus presence promotes antibody production against the virus. 9 MLV vaccines were listed in Murtagh's 2011 review of current PRRSV vaccines [67]. In Reunkaradhya's more recent 2015 review, the MLV vaccines are efficient against regional strains, but induce less protection against new emerging strains or in various other regions. Upon vaccination with commercially available live attenuated vaccines, pigs have shown protection against PRRS viruses of the same or closely related strains. However, reinfection with a more

genetically diverse strain can still occur due to the lack of heterologous protection [66]. The protection from MLV has been described as a reduction in clinical symptoms, viremia, and shedding; however, complete sterilizing immunity has not been described to date [86]. Additionally, the current live attenuated vaccines do not provide the ability to differentiate between a vaccinated pig and an infected one [40, 115]. Several new vaccine approaches are being evaluated, but they fail to provide complete sterilizing protection [86]. These new approaches include but are not limited to modified live virus generated by DNA shuffling and codon pair de-optimization and the use of virus vectors to deliver PRRSV proteins [86].

cDNA clones provide opportunities to study PRRSV replication and gene function as well as provide an opportunity to be used for vaccine development. The cDNA clones can be mutated with insertions or deletions. The change in replication rates, phenotype changes, or viability is what is studied to give researchers an idea of possible solutions in vaccines, but is also a concern, because PRRSV is a RNA virus. This means that in creating the cDNA clone, the mutation can result in the vaccine reverting back to its infectious self [86].

A cDNA clone can be used for a vaccine such as a DIVA marker vaccine. DIVA stands for Differentiating Infected from Vaccinated Animals. cDNA clones in DIVA vaccines are used to delete a specific sequence of the viral genome being protected against. DIVA vaccination strains eliminate a B-cell epitope. One obstacle is the ability to produce an assay able to detect the antibodies binding with the epitope missing from the vaccination. Compliance marker vaccines also use cDNA clones by using one gene from a strain of PRRSV and inserting the gene into another strain of PRRSV. This results

in a chimeric PRRS virus where a non-virulent strain can minimize the virulence of another strain [35, 41]. The levels of protection and efficacy of marker vaccines are still being explored [86].

DNA shuffling is the production of chimeric DNA such as a cDNA clone, except by a different method. The DNA is made into fragments and piece back together to express only certain characteristics [74]. The advantage of this over a cDNA is the newly synthesized DNA has been seen to help create neutralizing protection among several strains [125]. This helps with cross-protection a common problem in vaccine approaches against PRRSV [86].

Outbreaks of mutated strains are re-emerging and a current vaccination known as SAVE is hypothesized to prevent PRRSV outbreaks by attenuating translation. SAVE, synthetic attenuated virus engineering, has been successful with polio and influenza, which give hopes to PRRSV [16, 65]. Codon pair de-optimization uses the advantage of genetic degeneracy by altering the 1 or 3 nucleotides that make up a codon creating a silent mutation. The same amino acid is produced, but translation is decreased which can lead to attenuation of an emerging protein [74]. This vaccine approach is still being explored and tested for efficacy in pigs.

One common concern associated with many live virus vaccinations is the safety of its use. Because the virus is live and has the ability to replicate, the virus can cause side effects or revert back to the virulent strains and cause disease [86]. Therefore, several viral vector approaches that express structural proteins such as M protein and GP5 of the PRRS virus have been evaluated to produce PRRSV vaccines [20]. The

efficacy of viral vectored vaccines such as pseudorabies, adenovirus, transmissible gastroenteritis (TGEV) continues to be the substantial concern [86].

3. The Use of an Adjuvant in Vaccines

As stated in the immunology portion of this thesis, an adjuvant could enhance the cytokines and other innate immune factors that are required to activate the adaptive immune system. The adjuvants that have been used in PRRSV vaccine trials included IL-12, IL-2, and IFN-α.These cytokines helped activate IFN-gamma, which reduced viral load, but did not activate or enhance the humoral response [13].

C-di-GMP adjuvants show promise in producing strong mucosal immune responses. A mucosal immune response is needed to provide complete protection against the respiratory symptoms of PRRSV. Madhun demonstrated the variation of immune responses between intranasal vaccinations versus intramuscular vaccination while using the c-di-GMP adjuvant. Intranasal vaccination produced a higher Th1 response; whereas, intramuscular vaccination produced a higher Th2 response based on cytokine production profile [55]. The use of the adjuvant during intranasal vaccination amounted to high mucosal IgA and IgG. Intramuscular vaccination did not produce IgA suggesting that the c-di-GMP adjuvant is best administered intranasally to produce an effective mucosal immune response [30]

The 2’, 3’ cGAMP adjuvant is also capable of enhancing T cell response of the VLP vaccine. Gao, et al., demonstrated that the adjuvant stimulated antigen specific CD8 T cell response and enhanced the expression of IFN gamma, type I interferon, and IL-2 of DNA vaccines in mice [50]. It has been demonstrated that the 2’, 3’ phosphodiester links

of the adjuvant acts as a messenger and induces the type 1 interferon cytokine response [36]. The cGAMP binds to the DNA in order to start a STING signaling cascade activating various transcription factors, which increases expression of the cytokines [26, 30].

4. Application of VLPs as a Vaccine Candidate for PRRSV

Virus-like particles (VLPs) have a structure similar, but are not identical to a particular virus. VLPs do not contain the genome of the virus, which makes the particles non-infectious. To be infectious, a virus needs to be able to replicate its genome and make copies of itself based on the genome. This provides an advantage of its use as a vaccine, because VLPs cannot revert to an infectious stage; therefore, they are a safer alternative to the current commercially used MLV [76]. The VLPs can be made for viruses with envelopes, but are most effective for viruses without envelopes. VLPs can be made for DNA, single stranded RNA, and double stranded RNA viruses [76]. The VLPs can be generated by expressing the different combinations of viral structural proteins of PRRSV [19].

VLPs can also be used for the development of a corresponding diagnostic DIVA assay. Not only is the genome absent in VLPs, but other proteins of the virus are not expressed [76]. A diagnostic assay could be developed to identify an antibody response to specific viral proteins not expressed in the VLPs to identify presence of the virus in the pigs. Only the structural proteins chosen to be expressed in the VLPs cannot be used as a marker for DIVA assays. The importance of the DIVA assay is to help control the vaccination program. It's important to distinguish if the animal in suspicion is actually infected with PRRSV or if the animal was vaccinated and some other infectious agent is

causing the clinical symptoms present [19]. The VLPs provide many unique advantages based on how the VLPs are formed and which system is used to produce the VLPs.

VLPs are nearly identical in structure to the virus they mimic with an absence of specific proteins and DNA or RNA inside the virus. In order to be used as a vaccine, the VLPs need to be able to enter the host cell much like the virus in order to induce an immune response. VLPs have been found to activate toll-like receptors or pattern-recognition receptors. Both receptors help initiate the immune response by recognizing a foreign particle [4]. The VLPs have many viral structural proteins that may activate the toll-like receptors. This multivalent characteristic offers an advantage for using VLPs as vaccines. VLPs are also more likely to be captured by the antigen presenting cells like dendritic cells [19].

The VLPs used in this thesis were developed and expressed by a former graduate student in the lab [72]. To make the VLPs, plasmids expressing the important structural proteins were constructed. This included the M protein, N, GP5, and envelope protein specific to PRRSV. To make the plasmids, cDNA were first made from viral RNA. The M, N, GP5, and E gene were amplified by PCR, and PCR products were cloned into the pCAGEN plasmid. After verifying the gene expression from the plasmids, the genes were transferred into POET1 plasmids, which is the transfer plasmid for the flashback expression system. Recombinant baculovirus expressing the M, N, GP5, and E were generated by co-transfection of POET1 plasmids and baculoviral DNA in insect cells. This system was chosen based on the success of the HPV vaccine licensed and used commercially [72].

In order for VLPs to be expressed for viruses with envelopes, an expression system that can express multiple proteins must be developed. For this purpose, the baculovirus system was developed [76]. The research to use VLPs for a vaccine for Bluetongue Virus (BTV) was used as the pilot research to develop the baculovirus system [5]. Belyaev and Roy expressed VP2, 3, 5, and 7 of BTV by constructing recombinant baculoviruses, which were used to infect insect cells to form VLPs. Neutralizing antibodies were produced, and the VLPs were able to successfully provide some cross protection among various strains [87]. Insect cells provide a unique environment for expressing VLPs. The cells can be scaled up quickly and with less associated costs [72]. Insect cells are low maintenance to keep growing and are needed in large scale for mass vaccine production [19]. The insect cells are often used along with the baculovirus expression system [19, 72].

The baculovirus expression system has several advantages over other expression systems used to develop VLPs. One very important advantage is that baculovirus has a few susceptible hosts available. Therefore, the baculovirus is unlikely to be infectious to the animals that are being vaccinated with the VLPs [88]. Another advantage specific to the vaccine use of baculovirus is the ability to produce VLPs in a large scaled production system [57]. Several vaccine approaches and other VLP expression systems lack both efficiency and cost effectiveness to be produced on a large scale which is important in vaccine production. The baculovirus system was once thought to be among them until the licensing of the HPV vaccine. [19].

VLPs are being considered in several vaccine trials for mammalian viral diseases because of the proven success in both ability to be expressed and ability to produce an

immune response. Jain, Sahni, and team reviewed the progress of VLPs as vaccines since the approach has become popular [42]. Further research has indicated the recommended vaccine for Hepatitis B, a human disease, is a VLP manufactured vaccine with great success. Several researchers are studying the use of VLPs to vaccinate for other versions of HPV, influenza, ebola, and HIV [42]. Influenza is an enveloped virus with a complex structure. This makes the formulation of VLPs difficult to produce. However, VLPs have successfully been produced and expressed for influenza [76]. One particular study has shown that VLPs for influenza can be effective at producing a lasting mucosal response and strong memory recall [63]. The success and effectiveness across these diseases suggests a possibility and a need to explore VLPs further in animal diseases such as PRRSV.

VLPs have been successfully generated for PRRSV, although there is much diversity in the formulations of the VLPs. Uribe-Campero and team have tested VLPs using plants to develop a vaccine for PRRSV. The proteins used were M, N, and GP5. Their results indicated the VLPs were expressed and were able to develop an immune response in mice [102]. Also, another plant derived VLP vaccine was tested in both mice and ferrets, and a strong humoral immune response was detected [84]. Currently no research has been published to the best of my knowledge regarding the immunogenicity and protection efficacy of plant derived VLPs in swine. Another study showed that VLPs produced using the baculovirus system induced a specific antibody response with enhanced interferon gamma production in mice [69]. Murthy describes the formulation of PRRSV VLPs using a Hepatitis B antigen hybrid containing the PRRSV epitopes,

however, the in vivo studies with this PRRSV VLP formulation are currently unavailable [68].

VLPs are being explored as a vaccine approach for many human and animal viral diseases, but the immunogenicity and protective efficacy of PRRSV VLPs in pigs have not been studied extensively. Therefore, more studies are needed to better evaluate the feasibility of using PRRSV VLPs as an alternative vaccine approach. Additionally, it is important to study the effect of a novel class of adjuvant in enhancing the induction of mucosal immune response against PRRSV. The goal of this thesis project is to evaluate the immunogenicity and protective efficacy of PRRSV VLPs plus the novel 2,3-cGAMP adjuvant in pigs.

Chapter 2: Intranasal immunization of pigs with porcine reproductive and respiratory syndrome virus-like particles plus 2', 3'-cGAMP VacciGrade™ adjuvant exacerbates viremia after virus challenge*

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Background

Despite the use of live attenuated and killed vaccines, PRRSV is still the leading cause of porcine reproductive and respiratory disease complex and results in multi-million dollar losses annually in the U.S. [41]. This is partly due to the limited efficacy of killed vaccines and the lack of cross protection of live attenuated vaccines against heterologous virus strains [40, 66, 86]. Additionally, PRRSV infection displaying persistence in the host causes grave concern about using live attenuated vaccines for the control and prevention of this disease [2, 15]. Studies also show that vaccinating boars with attenuated vaccines adversely affects the quality of sperm, and PRRSV is still detectable in semen in vaccinated boars after virulent challenge [93]. Moreover, the severe atypical PRRS complex can occur in vaccinated animals, which will continue causing tremendous economic losses [59, 114]. Another major drawback of the current vaccines includes the inability to differentiate between vaccinated and naturally infected animals. Therefore, we urgently need novel vaccine approaches to overcome the limitations of these current vaccines.

Virus-like particles (VLPs) possess many attractive and desirable features of a potent vaccine candidate and have proven effective as a vaccine strategy against human papillomavirus (HPV) infections [12]. VLPs can be manufactured easily and quickly, which offers an advantage against a highly variable virus like PRRSV. Heterologous protection can be achieved by immunizing animals with a mixture of VLPs generated using the exact genetic match of the circulating virus strains. Scientists can easily differentiate vaccinated animals from naturally infected animals by RT-PCR or the absence of nonstructural protein specific antibodies. These properties support the notion that VLPs represent one of the most promising alternative vaccine strategies for the highly variable and persistent PRRSV.

PRRSV encodes seven structural proteins, which include nucleocapsid (N), membrane (M), envelope (E), glycoproteins 2, 3, 4, and 5 (GP2, GP3, GP4, GP5). Several previous studies

have reported the immunogenicity of PRRSV VLPs or chimera VLPs containing GP5 or GP5 and M proteins or the conserved protective epitopes of viral structural proteins in mice [68, 70, 102, 111]. Only one recent study described the partial protection against PRRSV in pigs immunized with PRRSV VLPs containing GP2, GP3, GP4, GP5 and M proteins [7]. More research is needed to fully explore the potential utility of VLPs in PRRSV vaccine development.

Type I interferon (IFN), the most important innate defense mechanism against virus infection, is essential to the induction of a robust adaptive immunity. Cyclic GMP-AMP, cGAMP, is an adjuvant currently being explored for its ability to increase type I interferon production. 2', 3' cGAMP is a cyclic dinucleotide, CDN, synthesized by cGAMP synthase (cGAS) and initiates signaling by first binding to a stimulator of IFN genes (STING) [36]. cGAS and STING are both necessary components for the 2', 3' cGAMP to be able to induce type 1 interferon production [95]. The IgA and IgG production in mucosal tissues after intranasal administration supports the use of this novel class of adjuvant in vaccines against respiratory disease [30].

In this study, we generated PRRSV VLPs by using the baculovirus expression system. The immunogenicity and protective efficacy of PRRSV VLPs with or without the use of the 2', 3' cGAMP were evaluated in pigs.

Materials and methods

Cells and recombinant baculoviruses

Sf9 insect cells (BD Biosciences) cultured in the TNM-FH Insect Cell Culture Media (BD Biosciences) were used in the generation of recombinant baculoviruses. TriEx™ Sf9 cells, serum-free adapted Sf9 derived cells, cultured in Novagen TriEx™ Insect Cell Media (Novagen, San Diego, CA) were used in the generation of VLPs by co-infection of cells with four separate recombinant baculoviruses. Recombinant baculoviruses were constructed using the FlashBac™ expression system (Oxford Expression Technologies). Briefly, PRRSV-23983 M, N, GP5, and E genes were first cloned into the transfer vector pOET1 [113]. After transfection of Sf9 cells with the transfer vector and the Flashbac baculovirus DNA using baculoFECTIN transfection reagents (Oxford Expression Technologies), recombinant viruses were harvested from the supernatant. The expression of M, N, GP5 and E from the recombinant baculoviruses were confirmed by Western blotting using HA tag specific monoclonal antibody (Sigma). Virus titers were determined by using the baculoQUANT ALL-IN-ONE™ Baculovirus DNA Extraction and Quantification Kit (Oxford Expression Technologies, Oxford, UK) following the manufacturer's instructions.

VLPs purification and characterization

TriEx SF9 cells were co-infected with recombinant baculoviruses containing PRRSV N, M, GP5, and E proteins at a MOI of 2 for N, GP5, and E proteins, and at a MOI of 3 for M protein with a starting cell concentration of 7.5 x 10^7 cells/ 50mL. Supernatants were harvested at 72 hours post infection. Cellular debris was removed by centrifuging at 2000 rpm for 10 minutes in a Beckman Coulter Allegra 6 centrifuge. The supernatant was then filtered through a 0.22μm filter and centrifuged at 28,000 rpm for 1 hour in a SW28 Rotor Beckman Ultracentrifuge. Pellets were resuspended in PBS and subjected to a discontinuous 15-60% OptiPrep® density gradient

(Sigma) centrifugation at 350,000 xg for 3 hours in a Beckman OPTIMA 130K Ultracentrifuge. The visible bands were collected and resuspended in PBS. Protein concentration was determined by using the Pierce® 660nm protein assay reagent (Thermoscientific Inc.) following the manufacturer's instructions.

For TEM study, the supernatant collected from the recombinant baculoviruses-infected TriEx SF9 cells were added to TSE Buffer containing 10mM Tris HCl, 1M EDTA, 100mM NaCl with 20% sucrose. After centrifugation at 350,000 xg for 1.5 hours 4°C in a Beckman OPTIMA 130K Ultracentrifuge, supernatant was discarded and the pellet was resuspended in 100µL phosphate-buffered saline. Samples were processed for TEM study as described previously [112].

Western blotting

TriEx Sf9 cells co-infected with recombinant baculoviruses containing PRRSV M, N, E, and GP5 gene were harvested at 72 hours after infection. Cells were lysed with a lysis buffer containing 0.01M Tris-HCl, 0.14M NaCl, 0.025% NaN3, 1% Triton X-100, and protease/phosphatase inhibitors cocktail (Thermo Scientific, Rockford, IL). Cell lysates and purified VLPs were subjected to SDS-PAGE gel electrophoresis (Novex by Life Technologies, Carlsband, CA) and transferred to a nitrocellulose membrane (Life Technologies, Gaithersburg, MD). The membrane was then blocked for 1 hour by rocking slowly at room temperature with 5% (w/v) milk powder in PBS plus 0.05% Tween 20 (PBST). Next, primary antibody, monoclonal mouse anti-HA antibody (Sigma-Aldrich, St. Louis, MO) diluted 1:5,000 in blocking buffer 5% (w/v) milk powder in PBST was added to the membrane and incubated overnight at 4°C on the rocker. The secondary antibody, goat anti-mouse IRDye (LI-COR, Lincoln, NE) diluted 1:10,000 in PBST was added to the membrane and incubated for 1 hour rotating at room temperature. Bands were visualized using the ODESSY Infrared Imaging System (LI-COR, Lincoln, NE).

Animal immunization and challenge

Eighteen two-week-old piglets as determined to be PRRSV negative by the IDEXX PRRS X3 Ab ELISA were purchased from a local farm. After acclimation for a week, piglets were randomly divided into three groups. One group served as a control and received 2 ml of PBS intranasally. Another group received 250 μg of PRRSV VLP. The third group received 250 μg of PRRSV VLP plus 83.3 μg of 2', 3'-cGAMP STING ligand (Invivogen). At two weeks after the primary immunization, a boost immunization of the same antigen preparation was administered intranasally. Two weeks after the boost immunization, all pigs were challenged with $2x10^5$ $TCID_{50}$ of PRRSV-23983. Clinical signs were observed and recorded daily. At day 0, 3, 7, and 10 after challenge, serum and nasal swabs were collected. At day 10 after challenge, all pigs were necropsied and lung tissues were collected. Rectal temperatures were taken at day 0, 3, 7, and 10 after virus challenge.

PRRSV specific antibody response

To measure the PRRSV N specific antibody response in the pigs, the serum samples were sent to the South Dakota State University Veterinary Diagnostic Laboratory for IDEXX PRRS X3 Ab ELISA. Data were presented as S/P ratios.

To measure PRRSV VLPs specific antibody response, the plates were coated overnight with the VLPs used in the vaccine at a concentration of 1μg/ml in 50μl of ELISA Coating Buffer (eBioscience, San Diego, CA.). The plates were washed 3 times with PBS containing 0.05% tween 20. The plates were then blocked with 100μl of 5% skim milk in PBS for 1 hour at room temperature. The plates were washed 5 times and the nasal samples in PBS were added at 100μl in each well. The plates were incubated 1 hour at room temperature. The plates were then washed 5 times and 100 μl of HRP conjugated anti-pig IgG or IgA was added to each well at a concentration of 1:10,000 for IgG and 1:100,000 for IgA. The plates are incubated at 37°C for 1

hour. Finally, the plates were washed 7 times and 100µl of substrate solution (1x TMB Substrate, eBioscience) was added. The plates were incubated for 15 minutes in the dark at room temperature. The reaction was terminated with 50µl of H_2SO_4 and read on a BioTek microplate reader at 450 nm.

Cytokine response

Quantitative ELISA assays were used to determine the concentrations of IFN-α, IFN-γ, and IL-10 in the serum samples. The ELISA protocol for IFN-α has been described in detail previously [123]. Briefly, the recombinant porcine IFN-α (PBL Interferon Source) serially diluted 1:2 starting with 800 units/ml was used to generate the standard curve. One hundred microliters of diluted standards and 100 µl serum samples in duplicate were added to the plate.

The IL-10 and IFN-γ ELISA were performed using commercial ELISA kits (Thermo Fisher Scientific) by following the manufacturer's instructions. One hundred microliters of standard was added to the appropriate wells at the appropriate dilutions in the standard diluent buffer. Fifty microliters of standard diluent buffer was added to each well containing 50 µl of the serum sample. The standard curve was used to determine the concentrations.

Real-time RT-PCR

Real-time RT-PCR was used to determine the viral RNA copies in the serum samples. Viral RNA was extracted from 200 µl of serum by using the high pure viral nuclei acid kit (Roche-Applied-Science). cDNA synthesis was performed by using a High Capacity cDNA Synthesis Kit (Applied Biosystems Inc.) according to the manufacturer's instructions. The forward primer (5' GTC AAT CCA GAC CGC CTT TA 3') and the reverse primer (5' GAT CAG GCG CAC AGT ATG AT 3') specific for the N gene of PRRSV were synthesized by the Integrated DNA Technologies. Real-time PCR was performed using the Brilliant II SYBR Green

QRT-PCR Master Mix (Agilent Technologies, Santa Clara, CA) and the ABI 7500HT Real-Time Thermocycler (Applied Biosystems, Foster City, CA). Ct values were recorded.

Statistical analysis

Student's t-test was used to analyze significance. A P-value < 0.05 was considered significant.

Results

Generation and characterization of PRRSV VLP

PRRSV VLP was generated from infection of insect TriEx™ Sf9 cells with recombinant baculoviruses expressing PRRSV M, N, E and Gp5 proteins. The expression of the viral proteins was detected in the cell lysates and purified VLP as shown in Figure 1. Furthermore, the formation and presence of VLP in the supernatants of infected cells was verified by the VLP visualized under TEM. The size of VLP was approximately 50 nm (Fig.1).

Immune response in pigs

To examine the antibody response to VLP, we first used the IDEXX ELISA to detect the N protein specific antibody since the VLP does contain N protein. As shown in Figure 2, we did not detect any significant difference in the antibody response before or after virus challenge, although a higher S/P ratio was observed prior to challenge in the VLP plus adjuvant group compared to the PBS and VLP groups. We used purified VLP as antigen to detect if any VLP specific antibody response was induced in the animals. We observed a significant increase in the OD reading for the VLP plus adjuvant group at day 7 after virus challenge when compared to day 3 after virus challenge, suggesting a boosting response elicited in the VLP plus adjuvant group for both IgG and IgA in the nasal samples. In contrast, no significant differences were observed for the PBS and VLP groups.

To examine the cellular response after virus challenge, we used ELISA to measure the interferon-γ in the serum. We found that all the animals showed a transient increase of IFN-γ at day 3 after virus challenge, followed by a decline at day 7 after virus challenge (Fig.3). No significant differences between the vaccinated and the control groups were observed.

The adjuvant is known to induce type I interferon to enhance the immunogenicity of vaccine candidates. We examined the IFN-α in the serum before and after virus challenge. IFN-α was detected in all groups prior to virus challenge (Fig.3). A significantly higher IFN-α response was seen in the VLP plus adjuvant group compared to the PBS group ($p < 0.05$) (Fig.3). A transient increase in the IFN-α at day 3 after virus challenge was only observed in the VLP and VLP plus adjuvant group (Fig.3). Although a slight increase in IFN-α was observed in the PBS group at day 3 and 7 after virus challenge, the level was mucher lower than the levels at day 0 after virus challenge in the VLP and VLP plus adjuvant groups. Furthermore, VLP induced a significantly higher IFN-α- than the PBS group at day 3 after virus challenge ($p < 0.05$). A higher IFN-α was also induced in the VLP plus adjuvant group at day 3 after virus challenge, but it is not significant when compared to PBS and VLP groups due to the high variability among the animals. The VLP plus adjuvant group did induce an overall higher IFN-α than the other two groups. This suggests that the VLP and VLP plus adjuvant delivered via the intranasal route have the capacity to activate type I interferon response in pigs.

PRRSV is known to induce IL-10 in infected animals. We examined the IL-10 in the serum using ELISA. We observed that no difference was found among the different vaccine groups in IL-10 response (Fig.3). Compared to the transient increase of IFN-α and IFN-γ in the challenged animals, IL-10 appeared to be more stable and did not show any increase or decrease from day 3 to day 7 after virus challenge. This suggests that IL-10 induction is independent of VLP and adjuvant used.

Rectal temperature, histological lesions, and viremia

No clinical signs of PRRSV infection were observed in the vaccinated and challenged animals. However, the rectal temperature of animals in the PBS control group was significantly higher than the VLP plus adjuvant group at day 10 after virus challenge (Fig.4). Similar

histological lung lesions were observed in animals of all groups. The overall lesion scores in the PBS and the VLP plus adjuvant group appeared to be slightly milder than the VLP group (Fig.4).

To quantify the viral RNA copies in the serum samples collected after virus challenge, we used qRT-PCR to detect the viral nucleocapsid gene transcript. We observed that none of the PBS vaccinated animals had detectable viral RNA at day 3 post challenge. While one out six animals in the VLP vaccine group and four out of six animals in the VLP plus adjuvant animals showed detectable viral RNA in the serum. At day 7 after virus challenge, 5 out of 6 animals in the PBS group demonstrated detectable viral RNA, while all 6 animals in the VLP and VLP plus adjuvant groups had detectable viral RNA. Furthermore, the viral RNA copies in the VLP and VLP plus adjuvant groups at day 7 after virus challenge were significantly higher than the PBS control group (Fig.4). The VLP plus adjuvant group was also significantly higher than the VLP group. At day 10 after challenge, three out of six animals in the PBS group showed detectable viral RNA, while two out of six from the VLP group and four out of six animals from the VLP plus adjuvant group had detectable viral RNA. No significant difference was observed between the groups at day 10 after challenge. The results suggest that the VLP and the adjuvant exhibited a synergistic effect on enhancing PRRSV replication in pigs.

Discussion

The genome of PRRSV contains 9 open-reading frames (ORFs). ORFs 2-7 encode viral structural proteins. Among the viral structural proteins, nucleocapsid protein (N, encoded by ORF 7), membrane protein (M, encoded by ORF 6), and glycoprotein 5 (GP5, encoded by ORF 5) are the most abundant structural proteins in the virions. These three proteins are essential to the formation of infectious virus particles for equine arterivirus, another member of the Arteriviridae [117]. Therefore, these three proteins likely play important roles in the assembly of virus particles for PRRSV, although other minor structural proteins including E (envelope protein), GP2, GP3, and GP4 (encoded by ORF 2, 3, and 4 respectively) may also contribute to the formation of infectious virus particles. A recent study has demonstrated a partial protection of PRRSV VLPs vaccine composed of five structural proteins including GP3, GP4, GP5, E, and M against homologous challenge when delivered together with PLGA nanoparticles [7]. In this study, we generated VLPs by expressing four PRRSV structural proteins including M, N, GP5, and E and assessed their immunogenicity and protective efficacy in pigs. No N protein and GP5 epitope specific antibody were detected in the serum at 2 weeks post boost, suggesting the VLPs dose and route of vaccination we used may not be sufficient in inducing an effective immune response. Alternatively, intramuscular injection could be a better choice of route of vaccination for VLPs. The reason we used intranasal delivery is because of recent studies showing cGAMP adjuvant as a mucosal adjuvant [8] and its superior performance than the intramuscular route when delivered together with antigens [56]. Nevertheless, we did observe a transient, but significant increase of VLPs specific IgG and IgA in the nasal samples of the VLPs plus adjuvant group at day 7 after challenge compared to day 3, but not in the PBS and VLPs groups. This result suggests that the adjuvant might be enhancing the immune response to VLPs. The significance of the VLPs specific antibody in protection remains to be determined. A higher viremia, but a lower rectal temperature, and milder histological lesions were observed in the VLPs plus the adjuvant group

compared to the VLPs group. To our knowledge, this is first report on the potential use of a new class of STING ligand as an adjuvant for domestic animals including pigs. Previous studies mainly focus on its potency in inducing type I interferon in mice to enhance the immunogenicity of vaccine candidates including VLPs [49, 56].

A previous study showed a discrepancy between PRRSV viremia and histological lesions and clinical signs under field conditions [21]. We observed a similar phenomenon in the vaccinated and challenged animals. There is no correlation between viremia and clinical signs and histological lesions. This raises the importance of screening clinical naïve pigs for virus shedding in the herds. The non-sympomatic animals may carry a relatively high viral load and contribute to the disease transmission and outbreaks.

Both VLPs and VLPs with the adjuvant enhanced the interferon-α response compared to the PBS control. One previous study showed that Ebola VLPs are capable of inducing type I interferon [3], which corroborate our findings here. The effect of the 2', 3' cGAMP adjuvant in triggering the induction of type 1 interferon has been well documented in other animal models [30, 96]. Our data provide evidence for its effect in type I interferon induction in pigs. We speculate that the activation of innate immune response by VLPs and the adjuvant may facilitate PRRSV replication which leads to enhanced viremia in animals from these two groups when compared to the control group. A recent study has indeed shown that influenza virus replication is promoted by the activation of toll-like receptor 7 (TLR7) and RIG-I activation in the respiratory tract [82]. This could be a common phenomenon for many mucosal related virus infections. Brockmeier et al. reported the role of type I interferon in enhancing the PRRSV specific interferon-γ response in pigs [9]. In our study, a transient increase in interferon-γ at day 3 after challenge was observed in all groups. We did not see the enhancement of interferon-γ response by the higher interferon-α induction in the VLPs and VLPs plus adjuvant groups. This could be due to the short time periods we collected the samples after virus challenge. Our data support an

earlier observation that PRRSV induces an early and transient interferon-γ activation [116]. Interestingly, IL-10 did not shown any change at day 3 and day 7 after virus challenge, suggesting that neither viremia nor interferon-α level affect IL-10 response in the animals.

Although we successfully generated PRRSV VLPs using the recombinant baculoviruses expressing Gp5, M, N and E proteins, we noticed the VLPs formation in the recombinant baculovirus co-infection model we used was not very efficient. This could be due to the protein combination we used is not ideal. Other minor structural proteins may be included in future studies to enhance the efficiency. Alternatively, strategies that can enhance the critical protein expression level should be considered to improve the VLPs formation efficiency as noted for human papillomavirus [89]. A multiBac expression system was proposed to enhance viral protein expression [89]. Contamination of baculovirus is another concern since it is difficult to remove all baculoviruses from purified VLPs. This has been described by others researchers [58]. We reason that the contaminating baculoviruses may serve as an additional trigger for type I interferon-α induction in VLPs vaccinated animals we observed. One previous study has indeed shown the effect of contaminating baculovirus in enhancing both innate and adaptive immune responses [58]. Another limitation of our study is that although Western blotting detected all four proteins from VLPs preparations, we could not be certain that all four proteins are actually incorporated into VLPs. Since all four proteins have the same HA tag, it would be difficult to differentiate them even by the immunogold TEM.

Overall, VLPs were generated from recombinant baculoviruses expressing Gp5, M, N and E proteins of PRRSV. The use of 2', 3'-cGAMP adjuvant in the intranasal vaccination of PRRSV VLP enhanced virus replication, but not disease severity in pigs. A higher level of interferon-α production, but not interferon-γ and IL-10, is correlated with enhanced virus replication. Future studies should focus on incorporating other viral proteins of PRRSV into VLPs and improving VLPs assembly efficiency. Additionally, different dose of VLPs, different

adjuvant and alternative route of vaccination such as intramuscular injection should be explored in the future to fully assess the feasibility of such a vaccine platform for PRRSV control and prevention.

Conclusions

Intranasal immunizations of pigs with PRRSV VLPs and VLPs plus the 2', 3'-cGAMP VacciGrade^{TM} adjuvant exacerbates viremia. A higher level of interferon-α production is correlated with enhanced virus replication. PRRSV VLPs and PRRSV VLPs plus the adjuvant fail to provide protection against PRRSV challenge.

Figure legends

Figure 1. VLPs generated from recombinant baculoviruses expressing PRRSV proteins. **Left Panel**: Detection of PRRSV Gp5, M, N, and E proteins from recombinant baculoviruses-infected insect cells and from purified VLPs by Western blotting. Lane S: Protein standard; Lane 1: Cell lysates; Lane 2: Purified VLPs. **Right Panel**: TEM pictures of PRRSV VLPs.

Figure 2. PRRSV N and VLPs specific antibody response. **Top Panel**: PRRSV N specific antibody response in the serum samples as detected by the IDEXX ELISA kit. **Middle Panel**: PRRSV VLPs specific IgG response in the nasal samples. **Lower Panel**: PRRSV VLPs specific IgA response in the nasal wash samples. Averages and standard deviations of 6 animals per group at 0 DPC, 3 DPC, and 10 DPC are shown. * indicates $p < 0.05$ between 3 DPC and 7 DPC samples of the VLPs with the adjuvant group.

Figure 3. Cytokines in the serum of immunized and challenged animals. **Top Panel**: Interferon-α concentrations in the serum samples. **Middle Panel**: IL-10 concentrations in the serum samples. **Lower Panel**: Interferon-γ concentrations in the serum samples. Averages and standard deviations of 6 animals per group run in duplicate are shown. * indicates $p < 0.05$ between VLPs and PBS groups. ** indicates $p < 0.05$ between VLPs + Adj and PBS groups.

Figure 4. Rectal temperature, viremia, and histological lesions of lungs of animal challenged with PRRSV. **Top left panel**: Averages and standard deviations of rectal temperatures of 6 animals in each group at defined time points after virus challenge. * indicates $p < 0.05$ between PBS and VLPs & adjuvant group. **Top right panel**: Averages and standard deviations of Ct values of animals with detectable PRRSV RNA at duplicate qRT-PCR runs. * indicates $p < 0.000001$ between VLPs + Adjuvant group and PBS group. ** indicates $p < 0.01$ between VLPs and PBS groups. **Lower left panel**: representative pictures showing the histological lesions of

lungs at 10 DPC. Magnification 4X. **Lower right panel**: Averages and standard deviations of lung lesion scores of individual animals in each group. Scoring of the gross and microscopic lesions was based on the previously published data [38]. 0 = no lesions; 1 = mild interstitial pneumonia; 2 = moderate, multifocal interstitial pneumonia; 3 = moderate diffuse interstitial pneumonia; 4 = severe interstitial pneumonia.

Figure 1.

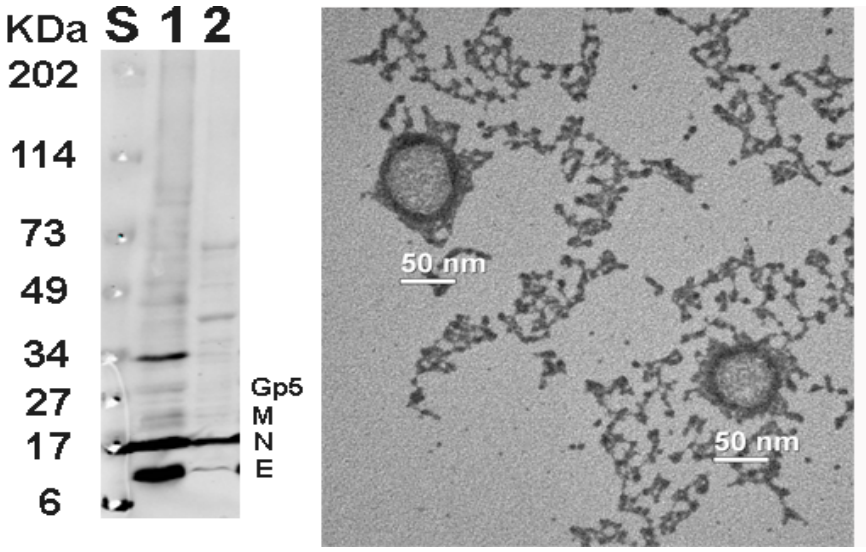


Figure 2.

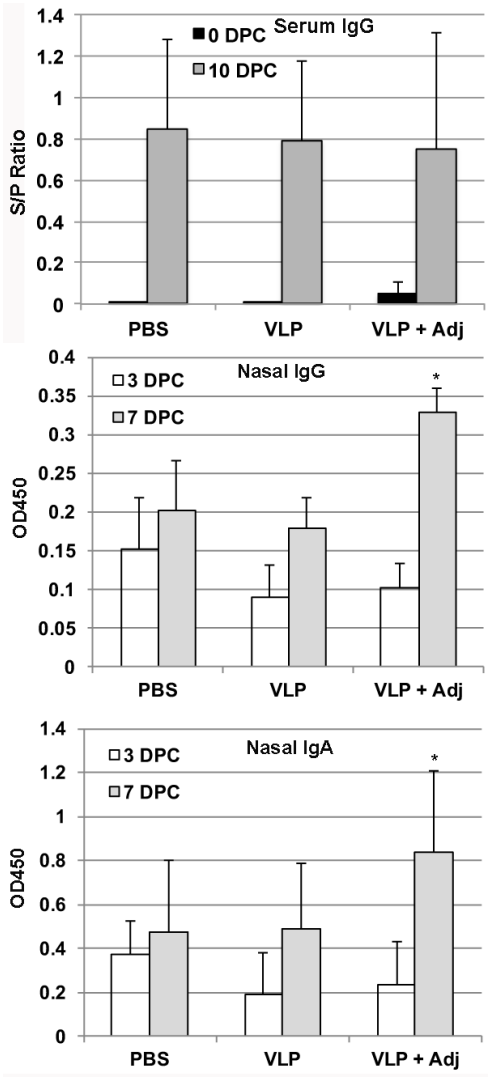

Serum IgG
0 DPC
10 DPC
S/P Ratio
1.4
1.2
1
0.8
0.6
0.4
0.2
0
PBS
VLP
VLP + Adj
Nasal IgG
3 DPC
7 DPC
OD450
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
*
PBS
VLP
VLP + Adj
Nasal IgA
3 DPC
7 DPC
OD450
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
PBS
VLP
VLP + Adj

Figure 3.

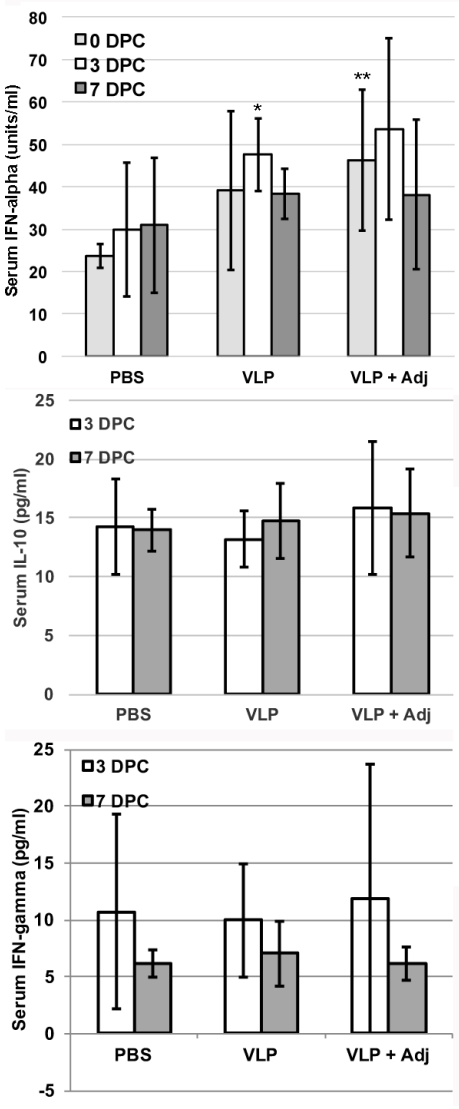
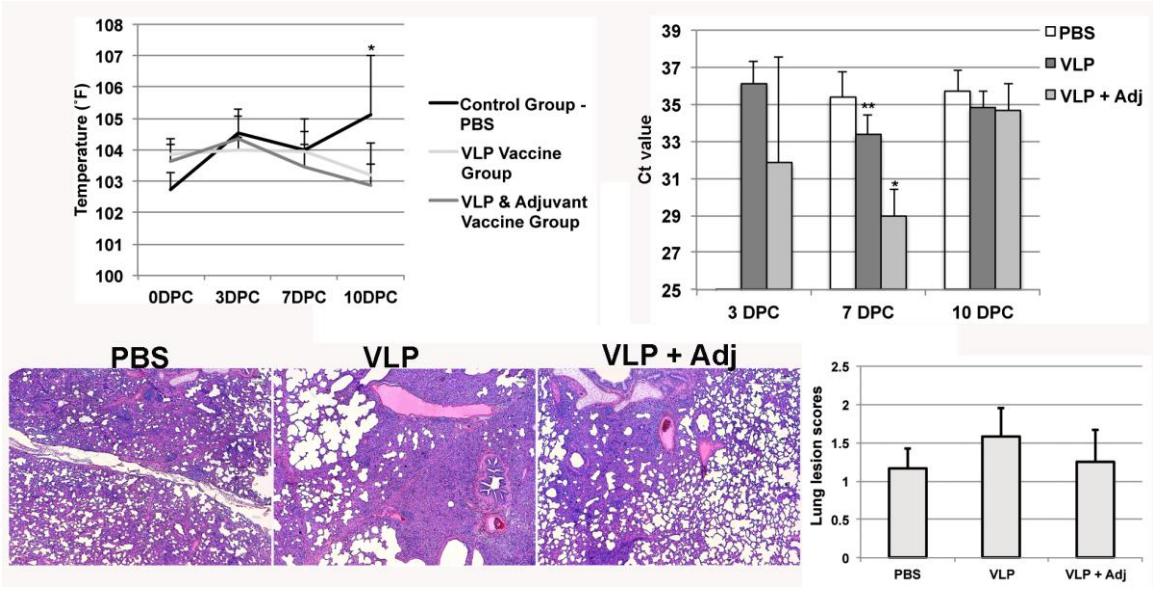

Serum IFN-alpha (units/ml)
0 DPC
3 DPC
7 DPC
*
**
PBS
VLP
VLP + Adj
Serum IL-10 (pg/ml)
3 DPC
7 DPC
PBS
VLP
VLP + Adj
Serum IFN-gamma (pg/ml)
3 DPC
7 DPC
PBS
VLP
VLP + Adj

Figure 4.


Temperature (°F)
108
107
106
105
104
103
102
101
100
0DPC
3DPC
7DPC
10DPC
Control Group - PBS
VLP Vaccine Group
VLP & Adjuvant Vaccine Group
Ct value
39
37
35
33
31
29
27
25
3 DPC
7 DPC
10 DPC
PBS
VLP
VLP + Adj
PBS
VLP
VLP + Adj
Lung lesion scores
2.5
2
1.5
1
0.5
0
PBS
VLP
VLP + Adj

Chapter 3: Summary and Conclusions

Porcine Reproductive and Respiratory Syndrome Virus continues to challenge farmers economically despite the circulating modified-live vaccine usage. Virus-like particles are a proven vaccine candidate that eliminates the ability of the virus to be infectious and allow farmers to distinguish between infected pigs and vaccinated pigs through the use of a DIVA assay. The adjuvant, 2,3, -cGAMP, has shown promise in enhancing the immune response of VLPs in mice. Here, we examined the immunogenicity and protective efficacy of PRRSV VLP in combination with the adjuvant in pigs.

The virus-like particle vaccine was composed of structural proteins, M, N, E, and GP5; however, we were unable to detect specific antibody to the N protein or the GP5 epitope prior to challenge. We suspect that the dose or the route of inoculation may contribute to the failure of VLP to induce a detectable immune response. The reason we used the intranasal route is because an enhanced mucosal response was observed in a previous study when compared to intramuscular administration [55]. After virus challenge, an increased interferon-alpha in VLP or VLP plus adjuvant group were detected, indicating that VLP and adjuvant primed the innate immunity prior to PRRSV challenge.

The VLP plus adjuvant group demonstrated a significant OD increase in IgG and IgA in the nasal samples when comparing day 3 and day 7 post challenge, suggesting a priming response to VLP occurred.

We observed increased viremia at 7 days post challenge for both vaccinated groups comparing to the control group. Upon 10 days post challenge a similar pattern

was seen; the VLP group had higher viremia than the control and the VLP plus adjuvant had a higher amount than the VLP group. The difference was not; however, statistically significant. The higher viremia contradicted the observations seen at necropsy. The VLP plus adjuvant group, which had the highest viremia at day 7, also had the lower rectal temperature and milder histological lesions. As this has been seen before in PRRSV field cases, we would recommend a need for more stringent screening processes in which pigs without clinical signs should be tested for PRRSV.

The adjuvant, 2'3'-cGAMP has been shown to increase type 1 interferon which is also confirmed by our study [96]. PRRSV is known to invade macrophages and other innate immune response cells [54]. Because the adjuvant is increasing the interferon-alpha, there is increased signaling to the immune system which could be bringing more cells for the virus to invade leading to the increased viremia. Interferon gamma also displayed an increase at day 3, but it was not significantly different among the groups. Both interferon alpha and interferon gamma decreased by day 7, not an uncommon response of the pig's innate immune system to PRRSV. IL-10 did not produce a noticeable difference between the groups or between day 3 and day 7.

In conclusion, the intranasal delivery of PRRSV VLP fails to induce an antigen specific immune response. The use of 2'3'-cGAMP adjuvant enhanced the innate immune response activated by the VLP, which may contribute to the increased viremia. Although a reminiscent IgA and IgG response to VLP were observed in the nasal samples at day 7 for the adjuvant, the antibody response did not reduce viremia. In the future, we should investigate the use of a stronger dosage, intramuscular administration, and

potentially the use of a different combination of viral structural proteins for VLPs to stimulate a potent and protective immune response.

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